Proc. Indian Acad. Sci. (Plant Sci.), Vol. 95, No. 6, December 1985, pp. 417–427.

Acquired cycloheximide resistance in *Neurospora crassa* and *Sclerotium rolfsii*

S B SULLIA and ROSE MARIA*

Department of Botany, Bangalore University, Jnana Bharathi Campus, Bangalore 560056, India

* Present Address: Department of Botany, NMKRV First Grade College for Women, Bangalore 560011, India

MS received 12 June 1985; revised 29 January 1985

Abstract. Acquired resistance to the antibiotic cycloheximide developed by *Neurospora crassa* and *Sclerotium rolfsii* was studied. Both the fungi gained certain level of tolerance to the antibiotic just after a single exposure and by serial transfers could adapt to the several-fold initial inhibitory dosage. Cycloheximide-resistance in both the cases was not a stable characteristic and was lost gradually on serial transfers in fungicide-free media. The resistant strains of both the fungi showed poor growth and decreased sporulation or sclerotia formation in fungicide-free media. *Sclerotium rolfsii* developed cross-resistance to Difolatan but not to Polyoxin-D, Hinosan and Bayleton. The cycloheximide-resistance in *Sclerotium rolfsii* was probably due to the conversion of cycloheximide into isocycloheximide which is a less toxic structural analog, as revealed by thin layer chromatographic studies of culture filtrates of resistant and sensitive strains. The resistant strain of *Sclerotium rolfsii* retained its pathogenicity to tomato, mustard and chilli seedlings. However, the loss of sclerotia forming capacity and the instability of the acquired resistance trait may prove to be of ecological disadvantage to the resistant strain.

Keywords. Acquired resistance; cycloheximide; *Neurospora*; *Sclerotium*.

1. Introduction

Cycloheximide (= Actidione) is a broad spectrum antifungal antibiotic isolated from *Streptomyces griseus* (Ford and Leach 1948). It is generally toxic to eukaryotes and is useful as a fungicide only when the host plant is less sensitive than the pathogen. However, it is widely used as a biochemical tool to block protein synthesis in eukaryotes. Though cycloheximide (CH) was considered as a specific inhibitor of protein synthesis, it has recently been shown to inhibit ribosomal RNA synthesis (Timberlake and Griffin 1974) and DNA synthesis (Sullia and Griffin 1977) in *Achlya bisexualis*. In view of the importance of CH, the present study on acquired resistance to CH in *Sclerotium rolfsii* and *Neurospora crassa* has been conducted. Earlier investigations have shown that some fungi gain resistance to CH, the extent of acquired resistance depending on the species (Salkin 1975; Griffin *et al* 1978; Sullia 1979). The mechanism of acquired resistance to CH has not been thoroughly understood. Although some work has been done with yeasts, filamentous fungi have scarcely been studied from the point of view of resistance.

2. Materials and methods

2.1

The test fungi were (i) *Neurospora crassa* Shear and Dodge-culture from Bangalore University Culture Collection No. 186, a saprobe (ii) *Sclerotium rolfsii* Sacc.-isolated

from damped-off mustard seedlings, also pathogenic to several other vegetable seedlings especially tomato and chillies.

The antibiotic fungicide cycloheximide (= Actidione) was bought from BDH.

2.2 *Radial growth of test fungi in CH-amended medium*

The test fungi were grown in PDA amended with CH ranging from 0·25–60 μg/ml and radial growth measurement technique was used for assessment of growth at 30°C. The ED_{50} value was determined by plotting the dosage-response curve. The level of resistance acquired due to first exposure to 0·5 μg/ml (*N. crassa*) and 40 μg/ml (*S. rolfsii*) was tested by a second transfer to CH-amended media. For other experiments, resistant strains were obtained by 7 serial subcultures in CH-amended medium.

2.3 *Determination of maximum concentration of antibiotic tolerated*

This was determined by serially subculturing the fungi in increasing concentrations of CH i.e., 1·5, 2·0, 2·5 and 3·0 μg/ml for *N. crassa* and 60, 80, 120, 160 and 200 μg/ml for *S. rolfsii.*

2.4 *Testing the stability of acquired resistance*

The CH-resistant strains of *N. crassa* and *S. rolfsii* were transferred to fungicide-free media through 7 subcultures and after each transfer the test organisms were reexposed to 0·5 and 40 μg/ml CH, respectively to check retention or loss of acquired resistance. The growth and sporulation of resistant strains were compared with those of sensitive strains, when returned to fungicide-free media and transferred to fungicide-free medium for 3 successive subcultures.

2.5 *Cross-tolerance studies*

Cross-resistance studies were conducted with *S. rolfsii* alone. Cross-resistance of CH-resistant strain towards Hinosan (5 μg/ml), Polyoxin-D (400 μg/ml), Difolatan (1,000 μg/ml) and Bayleton (200 μg/ml), was studied on amended PDA. The plates were incubated at 30°C for 20 days and colony diameters measured periodically.

2.6 *Assay of CH in culture filtrates of sensitive and resistant strains*

To determine the comparative ability of sensitive and resistant strains to convert CH into other compounds, mainly analogs of CH, the fungal culture filtrate after a definite period of growth was subjected to analysis by thin-layer chromatography (TLC) method detailed below. The CH-resistant and sensitive strains of *S. rolfsii* were transferred to replicates of 250 ml flasks containing 50 ml of PD-broth each and incubated at 28°C in a rotary shaker adjusted to 120 rpm. CH was introduced into each flask after 5 days of uniform mycelial growth in the flasks to obtain a concentration of 40 μg/ml.

Mycelial mats were filtered off with Whatman filter paper after 15 days. The culture filtrate was extracted with equal volume of chloroform and the chloroform layer was separated, evaporated to dryness and the residue redissolved in 1 ml of chloroform. This solution was used for chromatographic assay. Silica gel TLC plates were used for

Table 1. TLC of cycloheximide on silicagel. R_f values of cycloheximide and its analogs in the two solvents, ethyl ether and ethyl acetate.

Name of compound	R_f values	
	Ethyl acetate	Ethyl ether
CH	0·75	0·38
Iso-CH	0·80	0·67
CH-acetate	0·80	0·42
Streptimidone	0·58	0·38
CH-oxime	0·16	0·00
Streptovitacin-A	0·23	0·00
Anhydro-CH	0·80	0·52

spotting and the chromatograms were run with ethyl acetate and ethyl ether upto a distance of 8–10 cm. The plates were scanned under UV light for fluorescence and the spots were marked with a pencil. R_f values were calculated.

The R_f values of pure samples of CH and its analogs were determined on silica gel thin layer plates separately with ethyl ether and ethyl acetate. The analogs of CH used were Streptimidone, Streptovitacin-A, Iso-CH, CH-acetate, Anhydro-CH and CH-oxime (table 1).

The objective of the experiment was to determine whether the fungus had the ability to convert CH into any of the known structural analogs which were less toxic to fungi.

2.7 *Assessment of the virulence of the resistant strains*

2.7.1 *Pre-emergence damping-off*: Samples of sterilised soil were mixed with mycelial bits of resistant and sensitive strains of *S. rolfsii* and incubated for 48 hr. After 48 hr of incubation, seeds of tomato (*Lycopersicon esculentum* Mill.), chilli (*Capsicum annuum* L.) and mustard (*Brassica nigra* L. (Koch)) were sown. Observations were made on the 10th day for tomato and mustard and on the 15th day for chilli. The percentage mortality was calculated based on 3 replicate pots.

2.7.2 *Post-emergence damping-off*: 10-Day old seedlings of tomato and mustard and 15-day old seedlings of chilli in pots were treated with inocula of resistant and sensitive strains of *S. rolfsii* fed to soil. Seedling mortality was recorded on the 20th day for tomato and mustard and on the 25th day for chilli.

3. Results and discussion

3.1 *Sensitive and resistant strains*

N. crassa was highly sensitive to CH, the ED_{50} value being 0·5 μg/ml. Figure 1 shows that in the fungicide-free medium (control) the growth curves of both sensitive and resistant isolates were identical and steeply rising showing no lag phase. A longer lag phase and a flattening of the growth curve was evident with increasing concentration of antibiotic

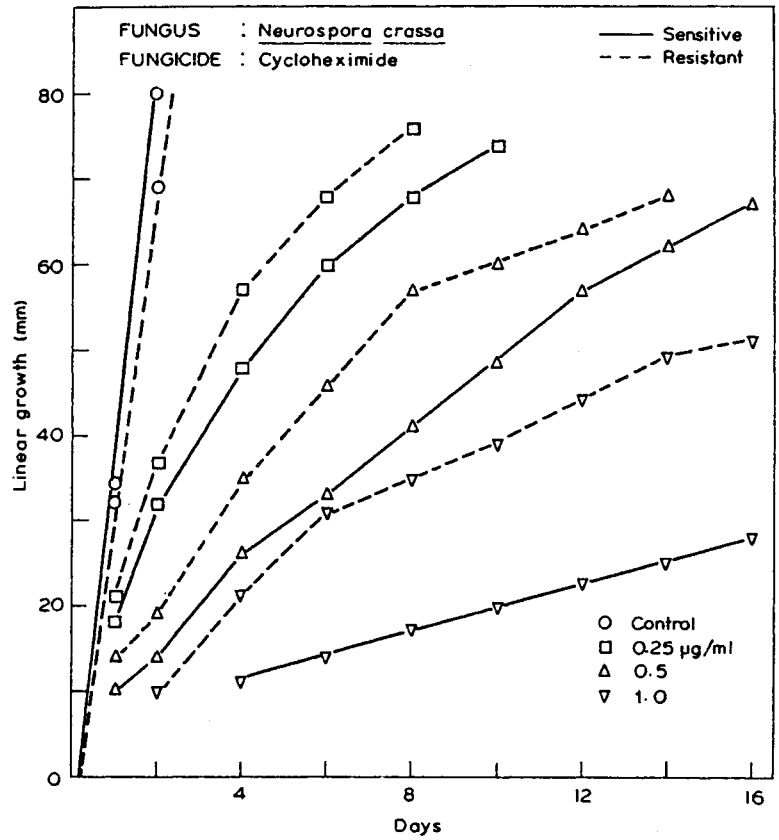

Figure 1. Linear growth of resistant and sensitive isolates of *N. crassa* in different concentrations of cycloheximide. Resistant isolate was priorly exposed to 0·5 µg/ml CH.

and this was more pronounced in the case of sensitive strains which showed much slower growth in all the 3 concentrations viz., 0·25, 0·5 and 1·0 µg/ml. On the 16th day the resistant isolate showed 45 mm colony diameter in 1·0 µg/ml compared to 25 mm of sensitive isolate. The adaptation of the fungus due to a single exposure to the antibiotic is evident from the experiment.

S. rolfsii was less sensitive to CH than *N. crassa*, the ED_{50} value being 20 µg/ml. The adaptation of the fungus to CH is quite evident on comparison of growth curves of sensitive and resistant isolates on medium containing 40 and 60 µg/ml CH (figure 2). At 60 µg/ml CH, the resistant isolate showed a steep growth curve, the fungus reaching a colony diameter of 65 mm in 8 days by which time there was no discernible growth of sensitive isolate. The resistance from single exposure was indeed very striking. Figure 2 also shows that the sensitive isolate plated on 60 µg/ml, after a prolonged lag phase slowly picked up growth and the curve showed an upward trend towards the end showing gain in tolerance during the prolonged maintenance of the fungus in amended medium. Adaptation of the fungus due to a single exposure has been reported in several species of fungi (Salkin 1975). Griffin *et al* (1978) have shown acquired resistance to CH in *Achlya* and they have also shown that some other fungi (e.g., *Cladosporium*) possess

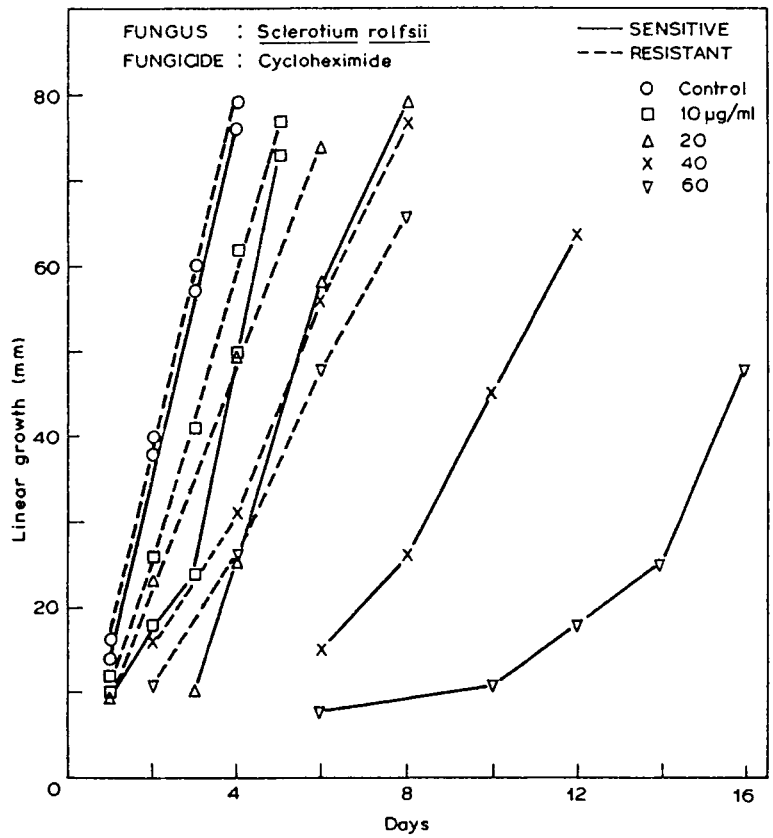

Figure 2. Linear growth of resistant and sensitive isolates of *S. rolfsii* in different concentrations of CH. Resistant isolate was priorly exposed to 40 µg/ml CH.

built-in resistance to CH. In the present study, *S. rolfsii* showed similar 'built-in' or 'constitutive resistance' to CH.

3.2 *Antibiotic level*

Through serial transfers from 1·5 µg/ml to 2·0, 2·5 and 3·0 µg/ml CH, *N. crassa* could be 'trained' to tolerate the antibiotic to some extent. The maximum concentration of the antibiotic to which the fungus could be trained was 2·5 µg/ml i.e., 5 times the ED_{50} dosage. Beyond that there was no discernible growth. Saprobic fungi are known to be more sensitive to CH than zoopathogenic fungi (Salkin 1975; Griffin *et al* 1978; Sullia 1979), and *N. crassa* is mainly a saprobic fungus.

The CH-resistant isolate of *S. rolfsii* was transferred from 60 µg/ml to 80, 120, 160 and 200 µg/ml CH. The sensitive isolate could not grow beyond 80 µg/ml. The trained isolate could grow at concentration upto 200 µg/ml. However, the resistant isolate produced no sclerotia beyond 100 µg/ml. Dharma Vir and Sharma (1980) have reported adaptation of *S. rolfsii* to benomyl to the concentration of 6,000 ppm whereas only 100 ppm could inhibit the growth of this fungus initially.

Table 2. The degree of retention of acquired CH-resistance of *N. crassa* and *S. rolfsii*. Radial growth in mm at 8th and 6th day respectively in test medium amended with 0·5 μg/ml and 40 μg/ml CH respectively.

N. crassa (8th day) Serial nos of transfers in fungicide-free medium								*S. rolfsii* (6th day) Serial nos of transfers in fungicide-free medium							
Cl.	1	2	3	4	5	6	7	Cl.	1	2	3	4	5	6	7
20	50	58	60	45	29	21	21	16	55	39	37	32	25	22	18

Cl, Control (unadapted)
1–7, Resistant strain after each transfer to fungicide-free medium.

3.3 *Stability of acquired resistance*

From table 2 it is clear that the resistant isolate of *N. crassa* retained its 'acquired resistance' to some extent during 5 serial transfers in fungicide-free medium. However, there was rapid loss of acquired resistance after the 6th transfer showing that acquired CH resistance is not a stable characteristic.

Somewhat similar result was obtained with *S. rolfsii* resistant to CH, where the acquired resistance was lost gradually (table 2). Ashida (1965) has shown that acquired resistance of *Sclerotinia fructicola* to copper and mercury was retained for 5 transfers but was completely lost by the tenth. Loss of CH-resistance has also been reported in certain saprobic and phytopathogenic fungi by Salkin (1975). When returned to fungicide-free medium there was reversion to initial levels of sensitivity, indicating that resistance to CH was not a genetic process but a purely transitory biochemical phenomenon. Grover *et al* (1961), showed in *Sclerotinia fructicola* and *S. laxa* that Actidione (= CH) resistance did not involve genetic selection and so the adapted isolates reverted back to parent types after growing for 2–5 generations in CH-free media.

3.4 *Growth of resistant strains*

The resistant strain of *N. crassa* on transfer to fungicide-free medium showed poor growth and no perithecia formation (table 3). This characteristic manifested for 3 successive transfers.

Resistant strain of *S. rolfsii* showed slower growth and poor yield of sclerotia compared to parental isolate in fungicide-free media (table 3). Dekker and Gielink (1979) demonstrated in laboratory tests that increased resistance to Pimaricin in selected strains of *Cladosporium cucumerinum* and *Fusarium oxysporum* f.sp. *narcissi* was associated with decreased radial growth and sporulation in vitro. Pimaricin, however, is an antifungal antibiotic chemically unrelated to CH. This shows that loss of sporulating ability in fungi may be induced by a large number of antifungal agents irrespective of their mode of action.

3.5 *Cross-resistance*

CH-resistant strain of *S. rolfsii* showed negative cross-resistance phenomenon towards Polyoxin-D and Hinosan as the sensitive strain fared better than resistant in media

Table 3. Growth and sporulation of CH-resistant strain of *N. crassa* and *S. rolfsii* in fungicide-free medium as manifested during 3 successive transfers.

	Linear growth at 48 hr (in mm)		Ave. no. of perithecia/ sq. cm area after 7 days	Ave. no. of sclerotia/ plate after 7 days
	N. crassa	*S. rolfsii*	*N. crassa*	*S. rolfsii*
S	89	38	84	39
R	13	33	0	315

S, CH sensitive; R, CH resistant.

Table 4. Cross-resistance of CH-resistant strain of *S. rolfsii* to other antifungal agents.

Incubation time (days)	Fungal strain	Radial growth (mm)				
		CH	D	H	DF	B
5	S	10	20	56	5	8
	R	68	14	40	19	10
10	S	45	30	80	5	11
	R	80	26	66	59	17
15	S	*	53	*	5	15
	R	*	48	*	66	22
20	S	*	70	*	5	31
	R	*	64	*	80	36

CH, Cycloheximide; D, polyoxin-D; H, hinosan; DF, Difolatan; B, Bayleton; S, CH sensitive; R, CH resistant.
* Plates fully covered by fungal growth.

amended with these fungicides (table 4). In Difolatan, the CH-resistant strain covered 80 mm diameter on 20th day, whereas the sensitive isolate showed no growth indicating cross-resistance. There was also some degree of cross-resistance to Bayleton though not to the same extent as to Difolatan (figure 3). Even though, there was cross-resistance to Difolatan, there was no sclerotia formation. CH is an antibiotic which inhibits protein synthesis, whereas Difolatan interferes with the process of decarboxylation. The two obviously differ in their modes of action. Cross-resistance between totally unrelated compounds is relatively rare. Jurkowska (1962) showed cross-resistance to Zinc and Nickel salts in *Aspergillus niger* tolerant to copper sulphate.

3.6 *Conversion of antibiotic by resistant strain*

The extract of the culture filtrate of the CH-resistant strain in *S. rolfsii* showed one prominent spot in the chromatogram developed with solvent ethyl acetate (figure 4) which is identified as isocycloheximide (by referring to the R_f value given in table 1), which is less toxic than CH. Cycloheximide inhibits protein synthesis, DNA synthesis and

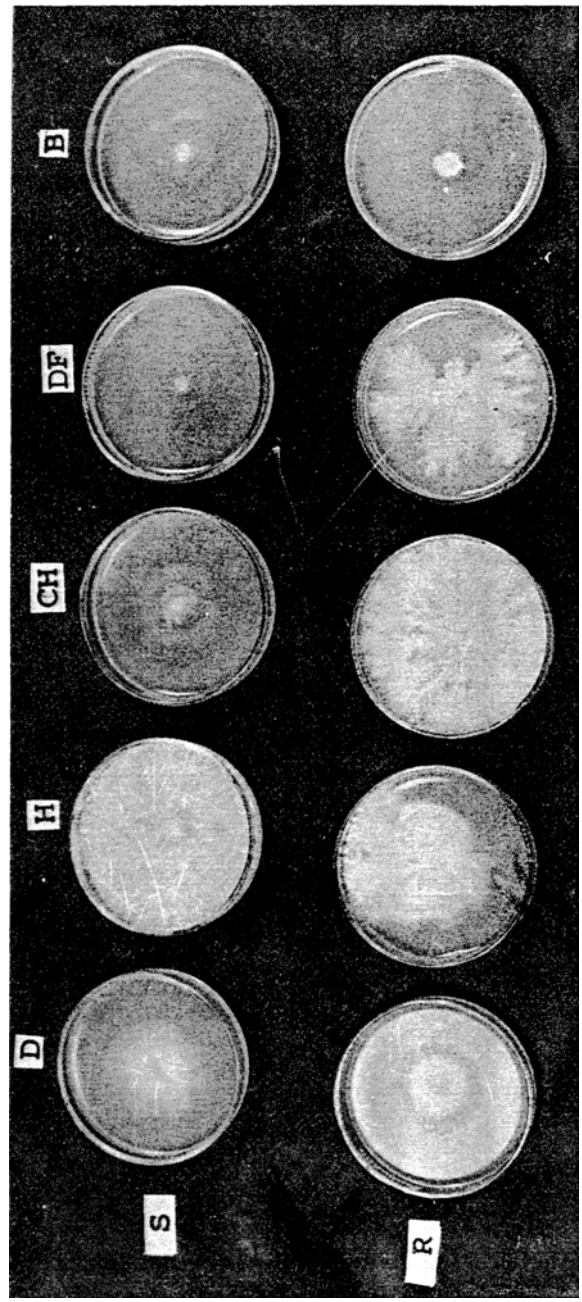

Figure 3. Cross-resistance of CH-resistant strain of *S. rolfsii* to different antifungal agents. D, Polyoxin-D; H, Hinosan; CH, Cycloheximide; DF, Difolatan; B, Bayleton; R, Resistant strain; S, Sensitive strain.

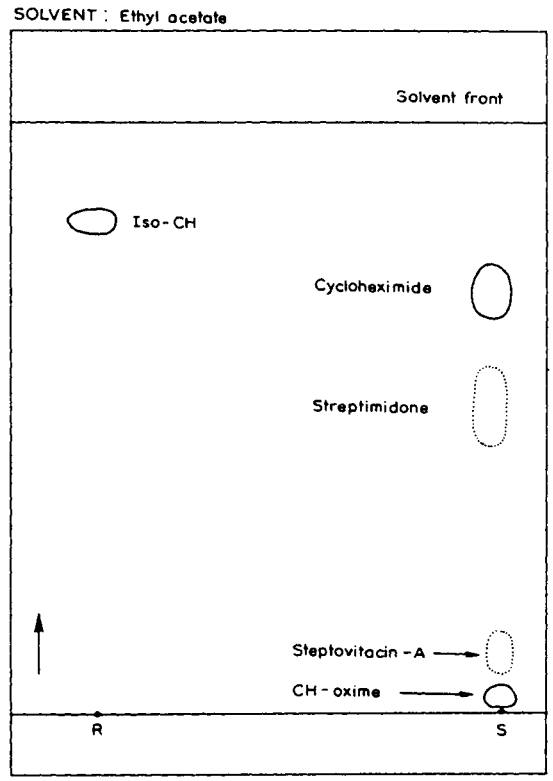

Figure 4. Diagram showing silicagel thin layer chromatogram of chloroform extracts of culture filtrates of CH-resistant and sensitive strains of *S. rolfsii* developed in ethyl acetate. The spots were marked by observing the fluorescence under UV light.

ribosomal RNA synthesis, whereas Iso-CH inhibits only one of the processes (Griffin 1981). The culture filtrate of sensitive strain of *S. rolfsii* showed several spots in addition to CH (figures 4 and 5). The spots were identified as Streptimidone, Streptovitacin-A and CH-oxime by comparing their R_f values with table 1. Isocycloheximide was present as a very faint spot. The sensitive strain retained most of the CH in the original form which is toxic to the fungus. The resistant isolate presumably metabolised almost all the CH into Iso-CH. Thus detoxification through conversion of the fungicide into a less toxic analog seems to be the main reason for resistance in this case. Somewhat similar results of conversion of a chemical by a fungus, but to a more toxic compound instead of less, has been reported. Gasztonyi and Josepovitis (1979) reported this with reference to the uptake and metabolism of triadimefon by fungi. A highly fungitoxic product (triadimenol) was formed to a high level in sensitive fungi, but it was very low or not found in resistant fungi.

3.7 *Pathogenicity*

The CH-resistant isolate of *S. rolfsii* caused a higher percentage mortality than sensitive in both pre and post-emergence stages in the test plants tomato, mustard and chilli (table 5). In an earlier experiment conducted in this laboratory (unpublished data), it

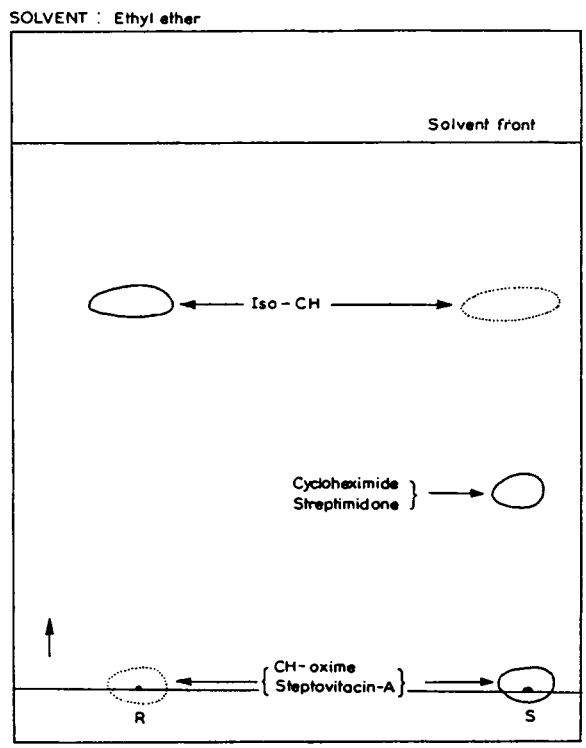

Figure 5. Diagram showing TLC of chloroform extracts of culture filtrates of CH-resistant and sensitive strains of *S. rolfsii* developed in ethyl ether. The spots were scanned through UV light.

Table 5. Pathogenicity of cycloheximide-resistant and sensitive strains of *S. rolfsii* to chilli, mustard and tomato seedlings (average of 3 replicates).

		Pre-emergence damping off			Post-emergence damping off		
Host	Fungal strain	Ave. no. of seeds sown/pot	Ave. no. of seeds emerged/pot	Percentage mortality	Ave. no. of seedlings initially present/pot	Ave. no. of seedlings dead/pot	Percentage of mortality
Chilli	Resistant	20	11·6	41·1[a]	14·4	4·4	30·4[b]
	Sensitive	20	13·0	35·0	16·6	3·3	19·9
Mustard	Resistant	20	8·6	56·6[b]	9·3	4·3	46·4[b]
	Sensitive	20	12·3	38·3	12·6	3·8	30·2
Tomato	Resistant	20	13·9	30·3[a]	18.6	5.3	28.5[b]
	Sensitive	20	14.9	25·3	17·6	2·9	16·9

[a] Difference between resistant and sensitive significant at 1% level as indicated by 't' test.
[b] Significant at 5% level.

was noticed that Polyoxin-D resistant strain of *S. rolfsii* and *Alternaria solani* had lost their pathogenicity and virulence. In this case, however, the resistant strain seems to be even more virulent than the original strain in the post-emergence stage. It has already been stated that the CH-resistant isolate lost its resistance to the antibiotic and its capacity to produce sclerotial bodies when transferred repeatedly to CH-free medium. This shows that though the fungus has not lost its virulence in the process of adaptation, it would, nevertheless, suffer from a disadvantage in competition as the adaptation is not a stable characteristic. The examples of fungi losing their acquired resistance or the phenomenon of reversion can be found in literature e.g., Kasugamycin resistance in *Pyricularia oryzae* (Miura *et al* 1975), Polyoxin-resistance in *Alternaria kikuchiana* (Kohmoto *et al* 1974) and benomyl-resistance in *Cercospora beticola* (Rupell and Scott 1974).

Acknowledgements

The authors are grateful to the Head of the Department of Botany, for laboratory facilities. SBS is grateful to UGC New Delhi, for sanction of a Research Project on the topic. RM acknowledges financial assistance from CSIR, New Delhi.

References

Ashida J 1965 Adaptation of fungi to metal toxicants; *Annu. Rev. Phytopathol.* **3** 153–174

Dekker J and Gielink A J 1979 Acquired resistance to pimaricin in *Cladosporium cucumerinum* and *Fusarium oxysporum* f. sp. *narcissi* associated with decreased virulence; *Neth. J. Plant Pathol.* **85** 67–73

Dharma Vir and Sharma R K 1980 Adaptation of *Sclerotium rolfsii* to systemic fungicide; *Curr. Sci.* **49** 285–286

Ford J H and Leach B E 1948 Actidione an antibiotic from *Streptomyces griseus*; *J. Am. Chem. Soc.* **70** 1223–1225

Gasztonyi M and Josepovitis G 1979 The activation of triadimefon and its role in the selectivity of fungicide action; *Pestic. Sci.* **10** 57–65

Griffin D H 1981 *Fungal Physiology*, (New York: John Wiley and Sons) p 383

Griffin D H, Sullia S B and Salkin I F 1978 Resistance of selected saprobic and zoopathogenic fungi to Cycloheximide; *J. Gen. Microbiol.* **105** 127–134

Grover R K and Duain Moore J 1961 Adaptation of *Sclerotinia fructicola* and *Sclerotinia laxa* to higher concentration of fungicides; *Phytopathology* **51** 399–401

Jurkowska H 1962 Investigation on the adaptability of *Aspergillus niger* to copper; *Bull. Ind. Acad. Gracove. Ser. B.* **4** 167–201

Kohmoto K, Miyake H, Nishimura S and Udagawa H 1974 Distribution and chronological population shift of Polyoxin resistant strains of black spot fungi of Japanese pear, *Alternaria kikuchiana* in field; *Ann. Phytopathol. Soc. Jpn.* **40** 220

Miura H, Ito H and Takahashi S 1975 Occurrence of resistant strains of *Pyricularia oryzae* to kasugamycin as a cause of the diminished fungicidal activity to rice blast; *Ann. Phytopathol. Soc. Jpn.* **41** 415–417

Ruppel E G and Scott P R 1974 Strains of *Cercospora beticola* resistant to benomyl in the USA; *Plant Dis. Rep.* **58** 434–436

Salkin I F 1975 Adaptation of Cycloheximide; in vitro studies with filamentous fungi; *Can. J. Microbiol.* **21** 1413–1419

Sullia S B and Griffin D H 1977 Inhibition of DNA synthesis by Cycloheximide and Blasticidin-S is independent of their effect on protein synthesis; *Biochim. Biophys. Acta* **475** 14–22

Sullia S B 1979 Acquired tolerance to antifungal agents in fungi; *Pestology* **3** 10–13

Timberlake W E and Griffin D H 1974 Differential effects of Cycloheximide and other inhibitors of protein synthesis on in vivo ribosomal RNA synthesis in *Achlya bisexualis*; *Biochim. Biophys. Acta* **353** 248–252